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Effect of Genetic Polymorphism of *CYP2A6* on Individual Susceptibility to Colorectal Tumors in Japanese Smokers*

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ABSTRACT

Tobacco smoking is a risk factor for colorectal cancer and adenomas. To clarify the effect of genetic factors on the risk for tobacco-related colorectal tumors in a Japanese population, we performed a case-control study on 300 patients with two or more tumors and 181 healthy controls; all were genotyped for *CYP2A6*4*, *CYP2A6*7* and *CYP2A6*9*. Cigarette smoking increased colorectal tumor risk (trend-test $P < 0.0000005$). Current smokers plus ex-smokers (ever-smokers) with the *CYP2A6*4/*4* genotype (whole gene deletion) showed the lowest risk among smokers [odds ratio (OR), 0.17; 95% confidence interval (CI), 0.05 - 0.62 compared to ever-smokers with the wild-type *CYP2A6*1/*1*]. When the participants were classified into four phenotype groups based on estimated CYP2A6 activity [*i.e.*, normal (**1/*1*), intermediate (heterozygotes for the **1* and a variant allele), slow (heterozygotes and homozygotes for variant alleles except for **4/*4*) and poor (**4/*4*)], the ORs (95% CIs) in ever-smokers of the normal, intermediate, slow and poor groups were 6.75 (2.73 - 16.76), 4.59 (2.10 - 10.06), 3.89 (1.69 - 8.95) and 1.17 (0.31 - 4.40), respectively, compared with never-smokers with normal CYP2A6 activity. The susceptibility to colorectal tumors was dependent on the predicted phenotype among ever-smokers (trend-test $P = 0.015$), but not among never-smokers (trend-test $P = 0.47$). Stratifying the subjects with respect to cumulative tobacco exposure and estimated CYP2A6 activity, we found the highest risk of colorectal tumors in subjects with higher CYP2A6 activity and higher cumulative tobacco exposure (trend-test $P = 0.000023$); the lowest risk was found in subjects with the lowest estimated CYP2A6 activity independent of tobacco exposure (trend-test $P = 1.00$). These results suggest that the gene-environment interaction (*i.e.*, the CYP2A6-smoking interaction) strongly affects the individual susceptibility to tobacco-related colorectal tumors.

Keywords: P4502A6; Tobacco Smoking; Colorectal Cancer; Colorectal Adenomas; Case-Control Study

1. Introduction

Colorectal cancer is now one of the most frequent causes of cancer mortality worldwide [1]. The etiology of colorectal cancer is complex and multifactorial, involving both genetic and environmental factors. Numerous case-control and cohort studies have been conducted to investigate the relationship between colorectal cancer and lifestyle factors, including the consumption of red or well-done meat [2], fat [3], and alcohol [4] and cigarette smoking [5]. Cigarette smoking has consistently been identified as a potential risk factor for colorectal adenomas [5], which are recognized as precursor lesions for most cases of colorectal cancer [6]. The association between colorectal cancer and smoking has recently been reported in many studies [5,7,8].

Tobacco smoke contains more than 60 carcinogens, including *N*-nitrosamines, polycyclic aromatic hydrocarbons (PAHs) and aromatic amines [9]. To exert their carcinogenicity, these carcinogens require metabolic activation, and this activation is mediated mainly by phase I drug-metabolizing enzymes. Among phase I enzymes, the cytochrome P450 (P450 or CYP) family plays a crucial role in this respect. CYP2A6, one of the major members of the P450 family in humans, is involved in the metabolic activation of carcinogens, particularly tobacco-related *N*-nitrosamines [10-12]. CYP2A6 also catalyzes the elimination of nicotine (this elimination is reported to be a major factor in the maintenance of smoking behavior and tobacco dependence [13]) by metabolizing nicotine to cotinine [14], and cotinine to trans-3'-hydroxy-cotinine [15]. Genetic polymorphisms of *CYP2A6* that influence the activity of CYP2A6 have been reported to affect

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tobacco-related lung cancer risk and smoking behavior [16].

To date, many variants of *CYP2A6* have been identified (URL: http://www.imm.ki.se/CYPalleles/cyp2a6.htm). Among these, the *CYP2A6*4* allele, a whole-gene-deletion polymorphism [17-19], is thought to be the most important allele in Asians because it has an allele frequency of up to 20%; however, its frequency is low in Caucasians [19,20]. Subjects homozygous for *CYP2A6*4* have been reported to show very low capacity to form cotinine from nicotine [21]. The *CYP2A6*7* allele causes an amino acid substitution (Ile471Thr) that leads to reduced enzyme activity [20]. The *CYP2A6*9* allele, which contains a −48T to G nucleotide substitution in the TATA box of the 5'-flanking region, has been reported to reduce the expression levels of CYP2A6 mRNA and protein in human livers [22]. Both the *CYP2A6*7* and *CYP2A6*9* alleles are also more common in Asians than in Caucasians [23].

Our recent studies have clarified that the mutant alleles described above reduce the susceptibility to squamous cell carcinoma and small-cell carcinoma, which are known to be types of lung cancer frequently induced by exposure to tobacco smoke [16,24,25]. The reduced risk in those with the *CYP2A6*4* allele was also seen in the reduced occurrence of oral cancer in betel/quid chewers with the same allele [26]. However, there is little information on the effects of *CYP2A6* polymorphisms on the risks for other smoking-related tumors, including colorectal cancer and adenomas.

In the present study, we found that *CYP2A6* polymorphisms, *CYP2A6*4*, *CYP2A6*7* and *CYP2A6*9*, reduced the tumor risk in combination with exposure to tobacco. We herein provide evidence that CYP2A6 activity is one of the principal factors that determine individual susceptibility to smoking-related colorectal cancer and adenomas.

2. Materials and Methods

2.1. Study Subjects and Data Collection

This study was approved by the ethics committees of the Osaka Medical Center for Cancer and Cardiovascular Diseases, the Osaka Central Hospital and Hokkaido University. All study participants were Japanese and were recruited from June 1993 to September 1997 at the Osaka Medical Center for Cancer and Cardiovascular Diseases. Cases were defined as patients with two or more colorectal tumors removed endoscopically within 3 months before recruitment. The colorectal tumors in this study included both adenomas (mild atypia, moderate atypia or severe atypia) and adenocarcinomas but not advanced or invasive cancer. Patients with one or more adenocarcinomas were assigned to the adenocarcinoma group, and those with adenomas only to the adenoma group. Individuals with a history of intestinal or gastric resection (other than appendectomy), familial adenomatous polyposis, ulcerative colitis, other malignant tumors and severe illness were excluded from the case group. Control subjects were healthy volunteers who required hospitalization for a health checkup. The ages of case and control subjects were defined at the time of the first hospital visit. Signed consent forms and completed questionnaires were collected from all case and control subjects before the collection of blood. The questionnaire covered smoking status (never-, ex- or current smoker), the total duration of smoking (excluding intermediate periods without smoking) and the average number of cigarettes smoked daily. The number of pack-year was calculated as a measure of cumulative cigarette smoking. One pack-year was defined as smoking 20 cigarettes daily for 1 year. Ex-smokers were defined as subjects with a minimum smoking history of 0.5 pack/day for at least 1 year. Current and ex-smokers were often combined into a group referred to as "ever-smokers".

2.2. Genotyping of CYP2A6

The *CYP2A6*4* allele genotyped in this study was the *CYP2A6*4A* variant, which is identical to *CYP2A6*4C* [20]. The genotyping of *CYP2A6*4* was based on polymerase chain reaction (PCR)/restriction fragment length polymorphism and was performed by a previously described method [20] with minor modifications. Briefly, a novel forward primer named 2A6-B6 (5'-CCT CAT CAC ACA CAA CTT CCT C-3') and a reverse primer named 2A6-UTRAS1 (5'-TGT AAA ATG GGC ATG AAC GCC C-3') [20] were used to amplify the common regions of *CYP2A6*1* and *CYP2A6*4*. The reaction mixture contained 1 × LA PCR Buffer II, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM 2A6-B6, 0.2 μM 2A6-UTRAS1, 0.5 U of LA Taq DNA polymerase (TaKaRa BIO, Shiga, Japan), and approximately 50 ng of genomic DNA in a final volume of 25 μL. PCR was carried out under the following conditions: initial denaturation at 94˚C for 3 min; followed by 30 cycles of denaturation at 94˚C for 30 s, annealing at 64˚C for 30 s and extension at 72˚C for 1.5 min; and subsequently a final extension at 72˚C for 3 min. The PCR products consisted of 1358-bp fragments from the *CYP2A6*1* allele and 1356-bp fragments from the *CYP2A6*4* allele; these fragments were digested with Eco81 I. Fragments of 824 bp and 728 bp were derived from the *CYP2A6*1* and *CYP2A6*4* alleles, respectively. The *CYP2A6*7* allele was genotyped using a two-step allele-specific-PCR method [20] in which the PCR products described above were used as templates.

For *CYP2A6*9* genotyping, a new method based on allele-specific-PCR was developed for this study. In the first PCR, a 440-bp fragment from the 5'-flanking region of exon 1 of *CYP2A6* was amplified using the primers

2A6 up-0.1 kb and 2A6 ex1R, as described previously [22]. In the second PCR, 2A6 TATA (5'-TCC CTC TTT TTC AGG CAG TAT-3') and 2A6 TAGA (5'-TCC CTC TTT TTC AGG CAG TAG-3') were employed as forward primers. The reaction mixture (25 μL) contained 1 μL of the first PCR products; 1 × PCR Buffer II, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM 2A6 TATA or 0.2 μM 2A6 TAGA, 0.2 μM 2A6 ex1R; and 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). Amplification was performed by initial denaturation at 94˚C for 1 min; followed by 20 cycles of denaturation at 94˚C for 30 s, annealing at 64˚C for 30 s and extension at 72˚C for 45 s; and subsequently a final extension at 72˚C for 1 min. An aliquot (5 μL) of second PCR products (387 bp) was analyzed by electrophoresis with a 1% agarose gel. Alleles that were not classified as *CYP2A6*4*, *CYP2A6*7* or *CYP2A6*9* were assigned to the wild-type *CYP2A6*1* allele.

In the analysis of putative CYP2A6 phenotypes, subjects were tentatively assigned to four groups based on their *CYP2A6* genotypes according to the definitions of Fujieda *et al*. [16]: the putative "normal" phenotype group, *CYP2A6*1/*1*; the "intermediate" group, those heterozygous for the *CYP2A6*1* allele (*CYP2A6*1/*4*, **1/*7* and **1/*9*); the "slow" group, those heterozygous or homozygous for variant alleles except for those homozygous for the *CYP2A6*4* allele (*CYP2A6*4/*7*, **4/*9*, **7/*7*, **7/*9* and **9/*9*); and the "poor" group, *CYP2A6*4/*4*.

2.3. Data Analysis

Differences in the distribution of relevant characteristics between cases and controls were evaluated using *t* tests and χ^2 tests. Compliance with Hardy-Weinberg equilibrium among controls was examined using the χ^2 test. Associations between susceptibility to colorectal tumors and smoking-related indicators or the *CYP2A6* genotypes/phenotypes were assessed using odds ratios (ORs). ORs and 95% confidence intervals (CIs) were calculated from logistic regression models to adjust for age and sex. When an association between smoking and colorectal tumor risk was detected, the never-smoker group was defined as the reference. The tertile values of continuous smoking variables (smoking duration, daily cigarette consumption and pack-year smoked) among ever-smokers in the controls were used as cutoff points. To investigate the impact of *CYP2A6* genotypes and the putative CYP2A6 phenotypes on colorectal tumor risk, the ORs among the never- and ever-smokers were estimated by comparing with the reference group of the never-smokers with the *CYP2A6*1/*1* genotype and the putative normal phenotype, respectively. Subsequently, among ever-smokers, the effects of the *CYP2A6* genotypes and putative CYP2A6 phenotypes on the risk were assessed, defining ever-smokers with the *CYP2A6*1/*1* genotype and the normal phenotype, respectively, as the reference groups. More detailed investigations were conducted in association with the combination of putative CYP2A6 phenotypes and cigarette smoking. The median values of smoking duration, daily cigarette consumption and pack-year smoked were used as the cutoff points.

To investigate whether the estimated risks were dependent on increased smoking variables, trend test was performed by assigning ordinal scores as continuous variables in the logistic regression models. When the median or tertile values of the smoking indicators among the control group were used as the cutoff points, *P* values for the trend were estimated with the ordinal scores 1 - 3 assigned to the three levels of smoking exposure (1 for never-smokers, 2 for light smokers and 3 for heavy smokers). When the trends of associations between putative CYP2A6 phenotypes and the risks were tested, ordinal scores of 1 - 4 were assigned to the normal, intermediate, slow and poor groups, respectively. All tests of statistical significance were two-sided. A *P* value of 0.05 was considered the threshold of significance. All statistical analyses were carried out by using the statistical software SAS, version 5.0 (SAS Institute, Inc., Cary, NC).

3. Results

The characteristics and smoking-related indicators of the colorectal tumor patients and healthy controls are summarized in **Table 1**. The mean ages of the case and control groups were significantly different ($P = 0.0048$). There more men than women in both case and control groups; however, the gender distributions were not significantly different between the two groups ($\chi^2 = 0.59$, $P = 0.44$). More ever-smokers (*i.e*., ex-smokers and current smokers combined) were present in case group than in the control group (72.3% versus 51.9%; $\chi^2 = 20.6$, $P = 0.0000058$). Ever-smokers in the case group had significantly longer durations of smoking ($P = 0.0013$) and higher values of pack-year smoked ($P = 0.000080$) than ever-smokers in the control group. No significant difference was found in the daily cigarette consumption between cases and controls ($P = 0.080$).

Table 2 shows the association between the various smoking-related indicators (smoking status, smoking years, daily cigarette consumption and pack-year smoked) and the risk for colorectal tumors. Ever having smoked cigarettes was associated with a significantly increased risk for colorectal tumors (OR, 2.95; 95% CI, 1.85 - 4.70). In particular, current smokers were at the highest risk (OR, 3.16; 95% CI, 1.90 - 5.26). The association with smoking was also examined in terms of smoking years, daily cigarette consumption and packyear. The highest exposure levels were associated with the highest

Table 1. Characteristics and smoking-related indicators of colorectal tumor patients and healthy controls enrolled in the present study.

	Cases (n = 300)	Controls (n = 181)	*P* value
Age, years			0.0048
Mean ± SD	56.1 ± 6.3	58.4 ± 10.1	
Median	57	58	
Range	41 - 67	35 - 82	
Gender, n (%)			0.44
Men	244 (81.3)	142 (78.5)	
Women	56 (18.7)	39 (21.5)	
Smoking status, n (%)			0.0000058
Never-smokers	83 (27.7)	87 (48.1)	
Ever-smokers	217 (72.3)	94 (51.9)	
Ex-smokers	85 (28.3)	42 (23.2)	
Current smokers	132 (44.0)	52 (28.7)	
Pack-year among ever-smokers			0.000080
Mean ± SD	35.1 ± 18.7	28.8 ± 17.8	
Median	33.8	25.4	
Smoking duration among ever-smokers, years			0.0013
Mean ± SD	27.6 ± 9.4	23.8 ± 9.7	
Median	30	20	
Cigarettes/day among ever-smokers			0.080
Mean ± SD	25.1 ± 11.5	23.1 ± 10.1	
Median	20	20	

Table 2. Association between cigarette smoking and risk of colorectal tumors.

	Cases (n = 300) n (%)	Controls (n = 181) n (%)	Adjusted odds ratio (95% CI)*	*P* value*
Smoking status				
Never-smokers	83 (27.7)	87 (48.1)	1.00 (reference)	–
Ever-smokers	217 (72.3)	94 (51.9)	2.95 (1.85 - 4.70)	
Ex-smokers	85 (28.3)	42 (23.2)	2.65 (1.52 - 4.59)	0.00055
Current smokers	132 (44.0)	52 (28.7)	3.16 (1.90 - 5.26)	0.0000091
Smoking duration, years†				
<20	29 (9.7)	26 (14.4)	1.53 (0.78 - 2.97)	0.21
20 to <30	74 (24.7)	35 (19.3)	2.41 (1.36 - 4.26)	0.0028
≥30	114 (38.0)	33 (18.2)	4.74 (2.70 - 8.32)	0.000000056
		Trend test‡	2.20 (1.69 - 2.85)	0.0000000030
Cigarettes/day†				
<20	44 (14.7)	27 (14.9)	2.08 (1.12 - 3.86)	0.021
20	72 (24.0)	37 (20.4)	2.53 (1.44 - 4.43)	0.0012
>20	101 (33.7)	30 (16.6)	4.42 (2.48 - 7.87)	0.00000046
		Trend test‡	2.11 (1.58 - 2.82)	0.00000047
Pack-year†				
<20	46 (15.3)	28 (15.5)	2.14 (1.15 - 3.99)	0.016
20 to <34	63 (21.0)	38 (21.0)	2.04 (1.17 - 3.58)	0.013
≥34	108 (36.0)	28 (15.5)	5.25 (2.93 - 9.41)	0.000000025
		Trend test‡	2.08 (1.61 - 2.70)	0.000000029

*Adjusted by logistic regression for age and gender; †Tertile values of ever-smokers among controls were used as cutoff points; ‡Based on a trend variable assigned values 1 - 4, including never-smoker (value 1).

risk, as shown by the groups smoking for ≥30 years (OR, 4.74; 95% CI, 2.70 - 8.32), smoking >20 cigarettes/day (OR, 4.42; 95% CI, 2.48 - 7.87) and hav- ing smoked ≥34 pack-years (OR, 5.25; 95% CI, 2.93 - 9.41). Elevated risks for colorectal tumors were signifycantly associated with increased smoking years, daily cigarette consumption and pack-years (trend-test $P = 0.00047$, 0.015 and 0.0026, respectively), even when the tests for trend excluded never-smokers.

To confirm the effects of *CYP2A6* genotypes on the risk for colorectal tumors in ever- and never-smokers, all case and control subjects were genotyped for *CYP2A6*1*, **4*, **7* and **9* (**Table 3**). The distribution of *CYP2A6* genotypes among controls were in Hardy-Weinberg equilibrium in ever-smokers ($\chi^2 = 2.81$, $P = 0.97$) and never-smokers ($\chi^2 = 5.74$, $P = 0.77$). This fact indicates that control subjects were sufficiently random and representative in both groups. Among never-smokers, no positive association was seen between *CYP2A6* genotype and the risk for colorectal tumors. Compared to never-smokers with *CYP2A6*1/*1* as a reference group, estimated risks among ever-smokers were significantly increased in *CYP2A6*1/*1*, **1/*4*, **1/*7*, **1/*9*, **4/*7*, **4/*9* and **7/*9* genotypes (e.g., *CYP2A6*1/*1*: OR, 6.85; 95% CI, 2.76 - 17.01). To investigate the impact of *CYP2A6* genetic polymorphisms among smokers only, ever-smokers with the *CYP2A6*1/*1* genotype were defined as the reference group. This analysis revealed that ever-smokers with the *CYP2A6*4/*4* genotype had a significantly reduced risk (OR, 0.17; 95% CI, 0.05 - 0.62). The ORs among ever-smokers with other genotypes tended to be lower than 1, but these ORs were not significant.

To further investigate the effects of *CYP2A6* genetic polymorphisms on the tumor risk in smokers, we classified all study subjects into four groups (normal, intermediate, slow and poor metabolizers) according to putative CYP2A6 phenotypes predicted from the *CYP2A6* genotypes, as previously defined by Fujieda *et al*. [16]. Among never-smokers, the putative CYP2A6 phenotypes did not affect the risk for colorectal tumors (trend-test $P = 0.47$, see **Table 4**). However, compared with CYP2A6-normal never-smokers, the ORs in predicted normal, intermediate and slow phenotype groups among ever-smokers were significantly increased to 6.75 (95% CI, 2.73 - 16.76), 4.59 (95% CI, 2.10 - 10.06) and 3.89 (95% CI, 1.69 - 8.95), respectively. However, compared with CYP2A6-normal ever-smokers, the estimated risk in CYP2A6-poor ever-smokers was significantly low (OR, 0.17; 95% CI, 0.05 - 0.62). These results clearly indicate that the risk of colorectal tumors in smokers is decreased by polymorphisms that reduce the activity of CYP2A6 (trend-test $P = 0.015$).

Table 4 shows the effects of putative CYP2A6 phenotypes on the risk of colorectal tumors with stratification by smoking-related indicators. The ORs among ex-smokers and current smokers relative to CYP2A6-normal never-smokers were significantly high, except for these in CYP2A6-poor groups. With respect to pack-years smoked, the highest OR was seen in groups with the putative normal phenotype with >25 pack-years smoked (OR, 10.03; 95% CI, 3.55 - 28.34) (see **Table 4**). In subjects with at least some CYP2A6 activity (*i.e*., all except

Table 3. Effect of *CYP2A6* genotypes on risk of colorectal tumors in never- and ever-smokers.

	CYP2A6 genotypes									
	**1/*1*	**1/*4*	**1/*7*	**1/*9*	**4/*4*	**4/*7*	**4/*9*	**7/*7*	**7/*9*	**9/*9*
Never-smokers										
Cases/controls*	16/24	13/13	10/6	17/13	5/5	7/7	9/5	2/5	1/4	3/5
Adjusted odds ratio (95% CI)†	1.00 (reference)	1.50 (0.54 - 4.16)	2.60 (0.77 - 8.77)	2.15 (0.80 - 5.77)	1.84 (0.44 - 7.68)	1.63 (0.46 - 5.72)	2.87 (0.79 - 10.38)	0.78 (0.13 - 4.72)	0.40 (0.04 - 4.00)	0.93 (0.19 - 4.58)
P value†	–	0.44	0.12	0.13	0.38	0.45	0.11	0.80	0.44	0.93
Ever-smokers										
Cases/controls*	52/15	36/16	24/13	44/15	5/8	12/6	15/6	7/2	13/6	9/7
Adjusted odds ratio (95% CI)†	6.85 (2.76 - 17.01)	4.59 (1.83 - 11.52)	3.56 (1.34 - 9.47)	5.65 (2.26 - 14.15)	1.19 (0.32 - 4.46)	3.81 (1.13 - 12.84)	4.80 (1.48 - 15.59)	5.40 (0.97 - 30.17)	4.56 (1.36 - 15.25)	2.36 (0.70 - 7.94)
P value†	0.000034	0.0012	0.011	0.00021	0.80	0.031	0.0090	0.055	0.014	0.16
Adjusted odds ratio (95% CI)‡	1.00 (reference)	0.67 (0.29 - 1.54)	0.52 (0.21 - 1.27)	0.83 (0.36 - 1.89)	0.17 (0.05 - 0.62)	0.56 (0.18 - 1.75)	0.70 (0.23 - 2.14)	0.79 (0.15 - 4.27)	0.67 (0.21 - 2.07)	0.35 (0.11 - 1.09)
P value‡	–	0.34	0.15	0.65	0.0071	0.32	0.54	0.81	0.48	0.072

*Never-smokers were made up of 83 cases and 87 controls, and ever-smokers were 217 cases and 94 controls; †Adjusted ORs for age and gender compared to never-smokers with *CYP2A6*1/*1* genotype as the reference group; ‡Adjusted ORs for age and gender compared to ever-smokers with *CYP2A6*1/*1* genotype as the reference group.

Table 4. Effects of putative *CYP2A6* phenotypes on risk of colorectal tumors in never-smokers and ever-smokers.

	Putative CYP2A6 phenotypes[*]				
	Normal	Intermediate	Slow	Poor	Trend test[†]
Never-smokers					
Cases/controls	16/24	40/32	22/26	5/5	
Adjusted odds ratio (95% CI)[‡]	1.00 (reference)	1.98 (0.88 - 4.43)	1.39 (0.58 - 3.32)	1.83 (0.44 - 7.66)	1.14 (0.79 - 1.65)
P value[‡]	–	0.099	0.46	0.41	0.47
Ever-smokers					
Cases/controls	52/15	104/44	56/27	5/8	
Adjusted odds ratio (95% CI)[‡]	6.75 (2.73 - 16.76)	4.59 (2.10 - 10.06)	3.89 (1.69 - 8.95)	1.17 (0.31 - 4.40)	
P value[‡]	0.000036	0.00014	0.0014	0.81	
Adjusted odds ratio (95% CI)[§]	1.00 (reference)	0.68 (0.35 - 1.34)	0.58 (0.27 - 1.21)	0.17 (0.05 - 0.62)	0.68 (0.50 - 0.93)
P value[§]	–	0.26	0.25	0.0070	0.015
≤25					
Cases/controls	13/7	30/25	22/11	1/4	
Adjusted odds ratio (95% CI)[‡]	3.39 (1.06 - 10.82)	2.29 (0.95 - 5.51)	3.78 (1.39 - 10.32)	0.42 (0.04 - 4.26)	0.86 (0.53 - 1.40)
P value[‡]	0.039	0.064	0.0094	0.46	0.54
>25					
Cases/controls	39/8	74/19	34/16	4/4	
Adjusted odds ratio (95% CI)[‡]	10.03 (3.55 - 28.34)	7.84 (3.29 - 18.68)	4.06 (1.62 - 10.20)	2.06 (0.43 - 9.78)	0.59 (0.30 - 0.90)
P value[‡]	0.000020	0.0000033	0.0029	0.36	0.014
Trend test *P* value[¶]	0.000023	0.00021	0.086	1.00	

[*]Putative CYP2A6 phenotypes: normal, *CYP2A6*1/*1*; intermediate, *CYP2A6*1/*4*, **1/*7* and **1/*9*; slow, *CYP2A6*4/*7*, **4/*9*, **7/*7*, **7/*9* and **9/*9*; poor, *CYP2A6*4/*4*; [†]Based on a trend variable assigned values 1 - 4 for putative normal, intermediate, slow and poor phenotypes, respectively; [‡]Adjusted ORs for age and gender compared to never-smokers with putative normal phenotype as a reference group; [§]Adjusted ORs for age and gender compared to ever-smokers with putative normal phenotype as a reference group; [¶]Based on a trend variable assigned values 1 - 3 for never-smoker, ever-smoker with ≤25 and >25, respectively.

those with *CYP2A6*4/*4*), the estimated risks for colorectal tumors were elevated with increased pack-years smoked (putative normal phenotype group, trend-test P = 0.000023; intermediate, P = 0.00021; and slow, P = 0.086). The ORs in subjects assigned to the putative normal or intermediate group were significantly dose dependent with regard to daily cigarette consumption and smoking duration (data not shown). In the CYP2A6-poor group, there was no evidence that the estimated risk was dependent on smoking-related indicators (pack-years, trend-test P = 1.00). Individuals who had smoked for >25 pack-years was strongly affected by the *CYP2A6* genotype (trend-test P = 0.014). In contrast, no significant association between the susceptibility to colorectal tumors and putative CYP2A6 phenotypes was observed in light smokers or never-smokers (trend-test P = 0.54 or P = 0.47, respectively). Thus, the combination of *CYP2A6* genetic polymorphism and cigarette smoking was clearly associated with the risk for colorectal tumors.

4. Discussion

Enzymes such as *N*-acetyltransferase [27-29], CYP1A1 [30,31], microsomal epoxide hydrolase [29,31-33], sulfotransferase [29] and glutathione *S*-transferase [28-30] are known to activate tobacco-related carcinogens (mainly PAHs and aromatic amines). Thus, a close association between genetic polymorphisms of these enzymes and susceptibility to colorectal cancer or adenomas is to be expected. However, few studies have provided definitive evidence that colorectal tumor risk is influenced by genetic polymorphisms of these enzymes, despite positive associations between the risk and smoking. The results of this current study are the first to show a clear association between *CYP2A6* genotypes and the risk of colorectal cancer and adenomas. Supporting this association, we previously reported that *CYP2A6* polymorphism is one of the principal determinants of the risks of tobacco-related lung cancer and betel/quid-related oral cancer [16,24-26].

Nowell *et al*. [34] reported a relationship between colorectal cancer risk and the urinary metabolite ratio of caffeine, which probably reflects the phenotype of CYP-2A6, although genetic variants were not analyzed. They demonstrated that subjects with high or medium putative CYP2A6 activity had an increased risk for colorectal cancer (high: OR, 2.9; 95% CI, 1.6 - 5.0 and medium: OR, 2.0; 95% CI, 1.0 - 3.7) compared with subjects with low activity. Their data strongly supported our concept that CYP2A6 could be one of the most important factors contributing to colorectal cancer risk. In contrast, Sachse *et al*. [35] reported that, in a Caucasian population, no significant association was seen between *CYP2A6* inactive alleles, particularly *CYP2A6*2*, and colorectal cancer risk (OR, 0.51; 95% CI, 0.28 - 1.06). This discrepancy between our results and theirs may be explained by several possibilities. First, ethnic differences exist in the frequencies of *CYP2A6* variants; the frequency of inactive alleles is lower in Caucasian than in Japanese populations [23]. The frequencies of the *CYP2A6*4*, *CYP2A6-*7* and *CYP2A6*9* alleles among controls in this study were 19.8%, 16.9% and 20.7%, respectively. These allele frequencies were similar to those previously observed in a large-scale Japanese population (χ^2 = 0.93, P = 0.82) [16] (in that study *CYP2A6*10* and *CYP2A6*11* alleles were classified as *CYP2A6*7* and *CYP2A6*1*, respectively) indicating that no selection bias was evident in our study population. In Caucasians, the frequencies of the *CYP2A6*4*, *CYP2A6*7* and *CYP2A6*9* alleles are reported to be 0.5%, 0.0% and 5.2%, respectively [23]. As a result, the statistical power is likely insufficient in the report by Sachse *et al*. [35]. Second, they analyzed the samples without distinguishing smokers from non-smokers [35]. We previously proposed that the positive relationship between cancer risk and CYP2A6 polymorphism can be detected only in smokers [16,20,25,26]. In accordance with our earlier studies, we found that CY-P2A6 polymorphisms altered the susceptibility to colorectal tumors in smokers but not in non-smokers (**Table 3**). Finally, most of the case subjects in the present study had colorectal adenomas, and most adenocarcinomas in the present study were cancer in adenomas; those with advanced or invasive cancer were excluded. The adenoma-carcinoma sequence is now widely accepted as a central pathway of carcinogenesis. Adenomas are recognized as the precursor lesions for the majority of cases of colorectal cancer [6]. The main environmental risk factors for colorectal adenomas are meat and fat consumption [36] and cigarette smoking [5], which are similar to those for colorectal cancer. However, it has been reported that the risk for colorectal adenomas was more strongly increased by cigarette smoking than was the risk for colorectal cancer. The effects of *CYP2A6* genetic polymorphism on the susceptibility to colorectal adenomas are thought to be clearer than those on the susceptibility to colorectal cancer.

We propose possible mechanisms for the correlation between *CYP2A6* genotypes and the susceptibility to smoking-related colorectal cancer. CYP2A6 is reportedly responsible for the metabolic activation of tobacco-related *N*-nitrosamines, including *N*-nitrosodiethylamine, *N*-nitrosonorntine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, *N*-nitroso-piperidine and *N*-nitrosopyrrolidine [11,12]. DNA adducts with these *N*-nitrosamines have been detected in human colonic tissues [37]. In addition, when human colon microsomes were incubated with known carcinogen methyl-*n*-pentylnitrosamine, the addition of coumarin, a typical substrate of CYP2A6, inhibited methyl- *n*-pentylnitrosamine metabolite formation [38]. These results suggest that CYP2A6 is expressed in human colon. We recently clarified that CYP2A6 mRNA and protein were over-expressed in colorectal adenocarcinoma. Kumarakulasingham *et al.* [39] examined CYP2A6 protein expression in normal colon and colorectal cancer tissues. Considering these lines of evidence together with our results, it seems reasonable to assume that CYP2A6 expressed in colon tissues is a factor critical for colorectal tumorigenesis.

In conclusion, the findings of this current study indicate that *CYP2A6* genetic polymorphisms alter the susceptibility to colorectal tumors in Japanese smokers, particularly in individuals highly exposed to tobacco smoke.

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Abbreviations

PAHs: polycyclic aromatic hydrocarbons;
CYP: cytochrome P450;
OR: odds ratio;
CI: confidence interval.

